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Applicants	: Jean-Marie Andrieu	
	: Louis Lu	
Title	: METHODS, AND COMPOSITIONS	Confirmation No.: 7112
	: FOR A THERAPEUTIC ANTIGEN	
	: PRESENTING CELL VACCINE	
	: FOR TREATMENT OF	
	: IMMUNODEFICIENCY VIRUS	

DECLARATION OF LOUIS (WEI) WU UNDER 37 C.F.R. 1.132

Mail Stop RCE
Commissioner for Patents
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Dear Sir:

I, Louis (Wei) Wu, hereby declare as follows:

1. I am a co-inventor of the subject matter claimed in U.S. Pat. App. No. 10/600,361 (hereinafter "the Application").

2. I have performed certain experiments relating to the expansion of HIV-pulsed dendritic cells, and the ability of these expanded cells to kill HIV-infected cells. Specifically, I compared the ability of dendritic cells expanded with thermally inactivated HIV and HIV which was chemically inactivated with aldrithiol-2 ("AT-2") to kill HIV infected cells. As shown in the attached Figure A, dendritic cells expanded with thermally inactivated HIV did not kill HIV infected CD4+T cells, whereas HIV inactivated with AT-2 did kill HIV infected CD4+T cells.

3. The experiments referred to in paragraph 2 above were performed as follows:

The HIV gag-specific CTL assay was performed in peripheral blood lymphocytes expanded by thermally or AT-2-inactivated HIV-pulsed dendritic cells (DCs), using HIV gag-pulsed autologous DCs as target cells. Non-pulsed autologous DCs were used as negative control targets while autologous DCs pulsed with recombinant hepatitis virus C (HCV) core protein (a gift of Dr. D. Han from Chinese Academy of Medical Sciences, Beijing, China) were used as nonspecific control targets. The percentage of specific cytolysis was calculated by subtracting the nonspecific ^{51}Cr release of the wells in the presence of recombinant HCV core-protein-pulsed autologous DCs.

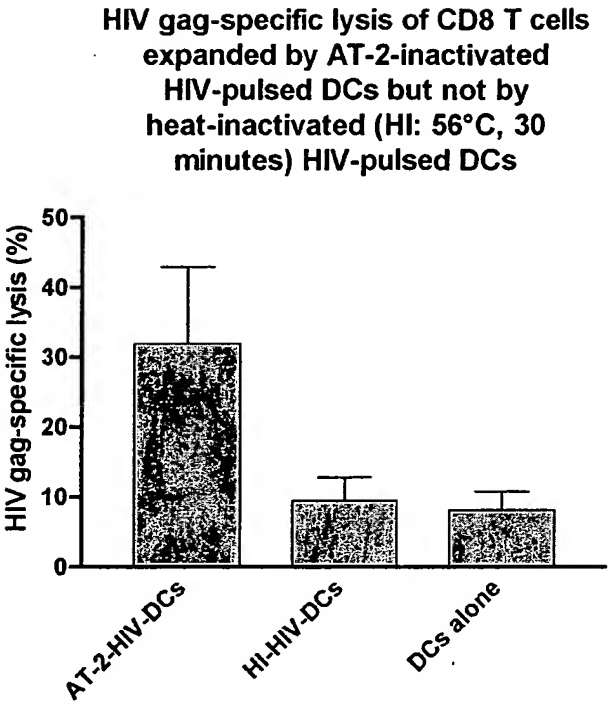
4. Figure A shows that the percent of HIV gag-specific lysis effected by heat-inactivated HIV-pulsed dendritic cells ("HI-HIV-DCs") was essentially the same as that effected by the control dendritic cells alone ("DCs alone"). In contrast, the AT-2 inactivated HIV-pulsed dendritic cells ("AT-2-HIV-DCs") killed a much higher percentage of cells than the control.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

23-02-2006

Date

Figure A


HIV gag-specific lysis of CD8 T cells expanded by AT-2-inactivated HIV-pulsed DCs but not by heat-inactivated (HI: 56°C, 30 minutes) HIV-pulsed DCs
HIV gag-specific lysis (%)
50
40
30
20
10
0
AT-2-HIV-DCs
HI-HIV-DCs
DCs alone

nature medicine

Therapeutic dendritic-cell vaccine for chronic HIV-1 infection

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We present the results of a preliminary investigation of the efficacy of a therapeutic dendritic cell (DC)-based vaccine for HIV-1. We immunized 18 chronically HIV-1-infected and currently untreated individuals showing stable viral loads for at least 6 months with autologous monocyte-derived DCs loaded with autologous aldrithiol-2-inactivated HIV-1. Plasma viral load levels were decreased by 80% (median) over the first 112 d following immunization. Prolonged suppression of viral load of more than 90% was seen in 8 individuals for at least 1 year. The suppression of viral load was positively correlated with HIV-1-specifc interleukin-2 or interferon-γ-expressing CD4+ T cells and with HIV-1 gag–specific perforin-expressing CD8+ effector cells, suggesting that a robust virus-specific CD4+ T-helper type 1 (T_H1) response is required for inducing and maintaining virus-specific CD8+ effectors to contain HIV-1 *in vivo*. The results suggest that inactivated whole virus–pulsed DC vaccines could be a promising strategy for treating people with chronic HIV-1 infection.

Although the natural immune response to human immunodeficiency virus type 1 (HIV-1) is not effective for eradicating the virus, vigorous HIV-1-specific CD4+ T_H1 cell responses have been shown to be associated with control of viremia and long-term nonprogression in infected individuals[1–6]. Early intervention with highly active antiretroviral therapy (HAART) during or shortly after acute infection was also associated with enhanced HIV-1-specific CD4+ T_H1-cell responses[7,8]. In contrast, at a later stage, HAART led to the decline of HIV-1-specific CD4+ T_H1-cell and CD8+ cytotoxic T lymphocyte (CTL) responses[2,9,10], suggesting that the functional capacities of HIV-1-capturing antigen presenting cells (APCs), which are required for the induction of the immune response, are progressively lost along the course of the infection. DCs, the most potent APCs, have a pivotal role in the initiation and maintenance of immune responses against viruses[11] and have been found to be impaired in individuals with progressive HIV-1 infection[12–15]. Recent studies have shown that the adoptive transfer of autologous DCs loaded *in vitro* with aldrithiol-2 (AT-2)-inactivated HIV-1 induced protective antiviral immunity in hu-PBL-SCID mice[16,17]. We had previously shown that a therapeutic vaccine made of AT-2-inactivated simian immunodeficiency virus (SIV) strain mac251 (SIVmac251)-loaded DCs led to considerable viral suppression in the absence of any antiviral therapy in Chinese rhesus monkeys that were immunized 2 months after having been infected with SIVmac251 (ref. 18).

Here, in a preliminary study, we explore the toxicity and the efficacy of a vaccine made of autologous monocyte-derived DCs pulsed with autologous AT-2-inactivated HIV-1 in patients with chronic HIV-1 infection.

RESULTS

Vaccination and its clinical consequences

We included 18 currently untreated HIV-1-infected individuals in the present study (Table 1). At day 0, we subcutaneously immunized each of them at the root of both arms and both thighs with a total dose (1 ml) of 3×10^7 quality-controlled AT-2-inactivated HIV-1-loaded viable DCs (see Supplementary Fig. 1 and Supplementary Tables 1 and 2 online). We administered two further injections with the same number of AT-2-inactivated HIV-1-loaded viable DCs at 2-week intervals. All patients completed the three doses of the therapeutic vaccine and were followed for 1 year thereafter without antiviral therapy.

The only clinical manifestation associated with the vaccine was an increase in the size of peripheral lymph nodes. The mean diameter (± s.e.m) of left and right axillary and inguinal lymph nodes increased from 0.33 ± 0.11 cm before the first immunization to 1.17 ± 0.20 cm ($P < 0.01$) at day 14 (second immunization), and to 1.61 ± 0.14 cm ($P < 0.001$) at day 28 (third immunization). The lymph node size remained significantly increased thereafter: 1.50 ± 0.19 cm at day 112 ($P < 0.001$), 1.67 ± 0.16 cm at day 224 ($P < 0.001$), and 1.06 ± 0.17 cm ($P < 0.01$) at 1 year. No local or systemic side effects developed and no clinical AIDS or milder immunodeficiency-related symptoms (such as weight loss, unexplained fever, chronic diarrhea or oral candidiasis) occurred during the study period.

Viral suppression after vaccination

The median plasma viral RNA load (PVL) of the 18 participants, which was stable for at least 6 months before immunization, decreased by 80% ($P < 0.01$) over the 112 d after the first immunization. It then remained stable until the end of the study (Fig. 1a–c).

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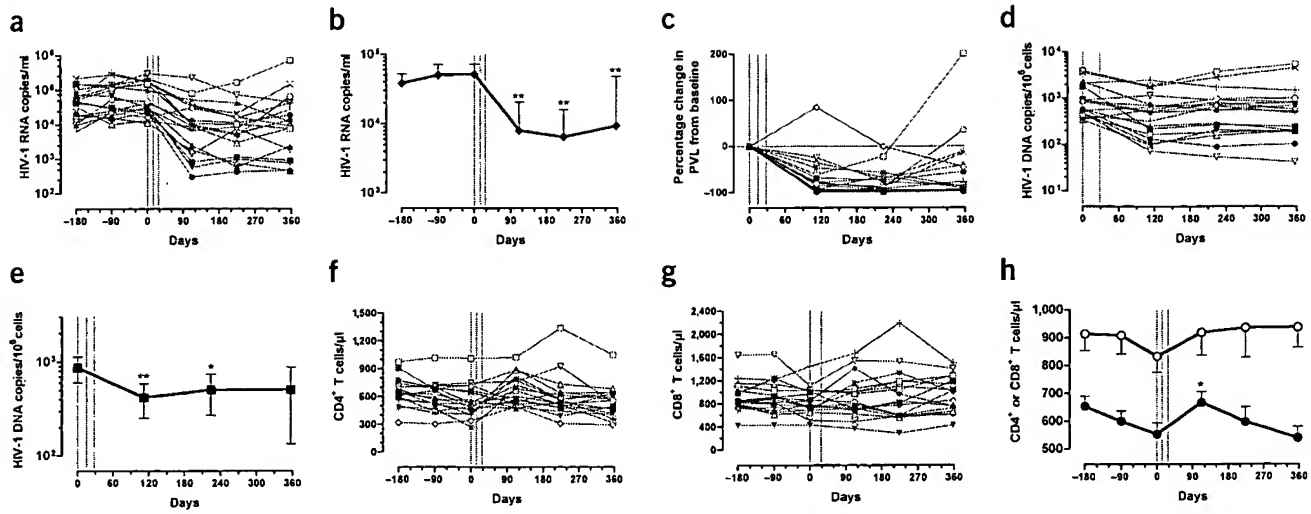

Figure 1 Immunologic and virologic evolution of the 18 immunized participants. (**a,b**) Individual or geometric mean (± s.e.m) (solid diamonds) plasma viral RNA loads (PVL) before and after immunization. Red lines indicate weak and transient responders who showed <90% PVL decrease by 1 year; green lines indicate strong and durable responders who showed >90% PVL decrease by 1 year. (**c**) Individual change of PVL by 1 year. (**d,e**) Individual or geometric mean (± s.e.m) (solid squares) of cellular viral DNA loads. (**f,g**) Individual blood CD4+ or CD8+ T-cell counts. (**h**) Mean (± s.e.m) CD4+ (solid circles) or CD8+ (open circles) T-cell counts of the 18 participants. $*P < 0.05$; $**P < 0.01$.

When looking at individual data (Fig. 1a,c), we observed that eight individuals' PVLs declined by more than 90%, whereas the response of the other ten individuals was weaker and transient (Supplementary Table 3 online). The median blood HIV-1 cellular viral DNA load of the 18 participants decreased by 50% ($P < 0.01$) over the first 112 d. This reduced cellular viral DNA load remained statistically significant ($P < 0.05$) at day 224 but became insignificant at day 357 (Fig. 1d,e).

The participants' blood CD4+ T-cell counts, which decreased (mean 17 cells/µl loss per month) during the 6 months preceding the immunization, increased significantly from day 28 to day 112 but

Table 1 Evolution of T-cell counts and plasma viral loads of 18 vaccinated individuals with HIV-1 infection

Participant ID	Sex	HLA A*0201	Age (years)	Months of known seropositivity	CD4 cells/µl				CD8 cells/µl				Plasma HIV-1 RNA copies/ml			
					–6 ms	–3 ms	Day 0	1 year	–6 ms	–3 ms	Day 0	1 year	–6 ms	–3 ms	Day 0	1 year
2	F	–	24	30	905	680	660	474	989	1,237	1,006	1,188	43,100	32,000	25,100	897
3	F	+	24	42[a]	631	584	490	460	796	677	793	760	10,000	18,100	33,100	2,277
4	F	+	23	28[a]	317	302	339	290	751	654	688	835	14,300	22,400	16,500	9,062
10	F	–	36	57	565	524	515	490	425	430	429	425	75,000	67,500	32,300	762
11	F	–	25	25	642	626	522	553	815	900	860	721	10,900	53,900	46,800	63,400
13	F	+	18	13	499	419	412	641	814	820	849	755	150,000	138,000	207,000	13,400
14	F	–	26	50	655	515	455	631	861	920	725	1,021	20,700	14,600	22,000	463
16	F	–	25	24	770	695	645	413	847	824	510	659	217,000	283,000	165,000	142,000
17	F	+	27	38	972	1,015	1,009	1,047	849	910	967	1,275	153,000	116,000	149,000	7,518
18	F	–	24	17	696	712	722	681	786	614	650	661	18,700	15,700	11,100	449
19	F	+	30	36	574	526	406	359	1,242	1,209	1,447	1,502	52,000	313,000	175,000	41,150
21	F	–	21	13	483	468	270	569	709	736	682	1,009	60,900	124,000	95,500	11,800
24	M	+	41	23	609	665	476	623	1,646	1,653	1,112	1,404	46,800	29,300	20,900	1,871
27	F	–	24	22	674	561	565	403	1,032	946	746	611	6,470	23,200	29,400	26,300
29	F	+	25	41[a]	773	694	528	648	821	789	746	624	17,600	25,500	41,600	18,400
31	F	+	29	65	594	439	492	314	1,111	1,028	1,035	1,288	52,800	103,000	211,000	696,000
32	F	+	29	18	738	684	721	586	1,152	1,141	1,036	1,132	33,800	10,200	13,200	7,778
33	M	+	27	17	699	721	746	533	827	877	735	1,029	105,000	141,000	300,000	43,740
Mean			27	31	655	602[b]	554[c,d]	540	915	909	834	916				
± s.d.			5	15	152	157	174	173	259	278	239	308				
Geometric mean													37,858	47,521	48,412	9,311[e]
± s.e.m.													16,677	19,851	20,844	38,267

[a]These patients had received antiretroviral drugs during the last 2 months (ms) of their pregnancy and were off therapy for ≥17 months. [b]Values at the time of inclusion (–3 ms) differed significantly ($P < 0.05$) as compared to preinclusion levels (–6 months). [c]Baseline values (Day 0, *i.e.*, 7 d after leukapheresis) differed significantly ($P < 0.05$) as compared to inclusion (–3 months) levels. [d]Baseline values (Day 0) differed significantly ($P < 0.05$) as compared to preinclusion levels (–6 months). [e]Values at 1 year differed significantly ($P < 0.01$) as compared to baseline (Day 0) levels.

returned progressively to baseline thereafter (Table 1 and Fig. 1f,h and Supplementary Table 4 online). No statistically significant changes were detected in the CD8$^+$ T cell count throughout the study (Table 1 and Fig. 1g,h).

HIV-specific immunity after vaccination

Total titers of antibody to HIV-1 remained unchanged following immunization (Supplementary Table 5 online). Neutralizing antibodies specific for autologous HIV-1 isolates were detected at low titers (1/10), the assay detection threshold in two individuals from day 0 to 1 year. They were undetectable in the 16 other participants, except transiently at low titers (1/10) in three patients (one at day 56 and day 112; two others at day 112 only).

Using a highly sensitive flow cytometry–based intracellular cytokine (ICC) assay (representative data from patient 13 in Fig. 2), we observed that HIV-1-specific interleukin-2 (IL-2)-expressing CD4$^+$ T cells increased around threefold ($P < 0.01$) over 1 year in the 18 immunized participants (Fig. 3a,b). HIV-1-specific interferon-γ (IFN-γ) expressing CD4$^+$ T cells increased around twofold ($P < 0.01$) (Fig. 3c,e), whereas HIV-1-specific IFN-γ-expressing (memory) CD8$^+$ T cells increased less than twofold ($P < 0.01$) with a peak increase at day 112 (Fig. 3d,e). Individual ICC data of the 18 immunized participants can be found in Supplementary Table 6 online. The 1-year PVL change was strongly correlated with the 1-year frequency of HIV-1-specific IL-2- (Fig. 3f) or IFN-γ-expressing CD4$^+$ T cells (Fig. 3g). The correlation of the 1-year PVL change with the 1-year frequency of IFN-γ-expressing CD8$^+$ T cells was much weaker (Fig. 3h).

Using the combination of antigen-specific human leukocyte antigen (HLA) tetramer binding and four-color flow cytometry in the 10 HLA-A*0201-positive participants (representative data from patients 13 and 19 in Fig. 4), we showed that HIV-1 gag–specific CD8$^+$ T cells increased more than threefold at day 112 ($P < 0.05$) and remained increased up to 1 year thereafter (Fig. 5a,b), whereas the percentage of HIV-1 gag–specific CD8$^+$ T cells expressing perforin (effectors) increased around twofold (Fig. 5c,d). By 1 year, there was no correlation between the percentage of gag-specific CD8 cells and the PVL change (Fig. 5e), whereas a significant linear correlation was observed between the percentage of HIV-1 gag–specific CD8$^+$ T cells expressing perforin and the PVL change (Fig. 5f). A strong correlation was observed at 1 year between the frequency of HIV-1-gag-specific perforin-expressing CD8$^+$ T cells and the frequency of HIV-1-specific IL-2-expressing CD4$^+$ T cells (Fig. 5g) or the frequency of HIV-specific IFN-γ-expressing CD4$^+$ T cells (Fig. 5h). The two individuals who had the highest percentage of virus-specific CD4$^+$ T cells expressing IL-2 by 1 year were the same as those who had the highest percentage of virus-specific CD4$^+$ T cells expressing IFN-γ (Fig. 5g,h). After removing these two individuals from the plot, the perforin-expressing gag-specific CD8$^+$ T cells still correlated significantly with the IL-2-expressing virus-specific CD4$^+$ T cells ($r^2 = 0.741$, $P < 0.01$) but did not correlate significantly with IFN-γ-expressing virus-specific CD4$^+$ T cells ($r^2 = 0.311$, $P = 0.149$).

To determine whether any initial biological parameter predicted the 1-year PVL response, we performed a correlation analysis between all initial parameters that we measured and the 1-year PVL change. Baseline PVL showed no correlation with 1-year PVL change (Supplementary Fig. 2 online). In contrast, the baseline CD4 cell-count correlated positively with 1-year decrease of PVL ($r^2 = 0.263$, $P = 0.029$) (Supplementary Fig. 2 online) and baseline HIV-1-specific IL-2-expressing CD4$^+$ T cells showed also a (marginal) positive correlation with 1-year decrease of PVL ($r^2 = 0.169$, $P = 0.089$; Supplementary Fig. 2 online). On the other hand, HIV-specific IFN-γ-expressing CD4$^+$ T cells, HIV-specific IFN-γ-expressing CD8$^+$ T cells, or the major maturation marker (CD83) of DCs did not correlate with 1-year PVL decrease (Supplementary Fig. 2 online).

DISCUSSION

This is the first demonstration in humans that a therapeutic vaccine made of autologous monocyte-derived DCs pulsed with autologous inactivated whole HIV-1 is capable of inducing an effective HIV-1-specific T-cell response associated with sustained viral suppression. Taking into account that over the 6 months before immunization, the PVL of the 18 participants remained stable, whereas their mean CD4 cell count decreased by 100 cells/μl (Table 1), the significant decrease of PVL as well as maintenance of CD4 cell counts observed at 1 year after immunization are particularly promising (Fig. 1). Although a nonspecific adjuvant effect of DCs cannot be ruled out, the key observation that enhanced CD4 T_H1 as well as perforin-mediated CTL responses elicited by the vaccine correlated with sustained viral suppression strongly supports an immunologic control of chronic HIV-1 infection by the therapeutic vaccine. We should emphasize, however, that the effi-

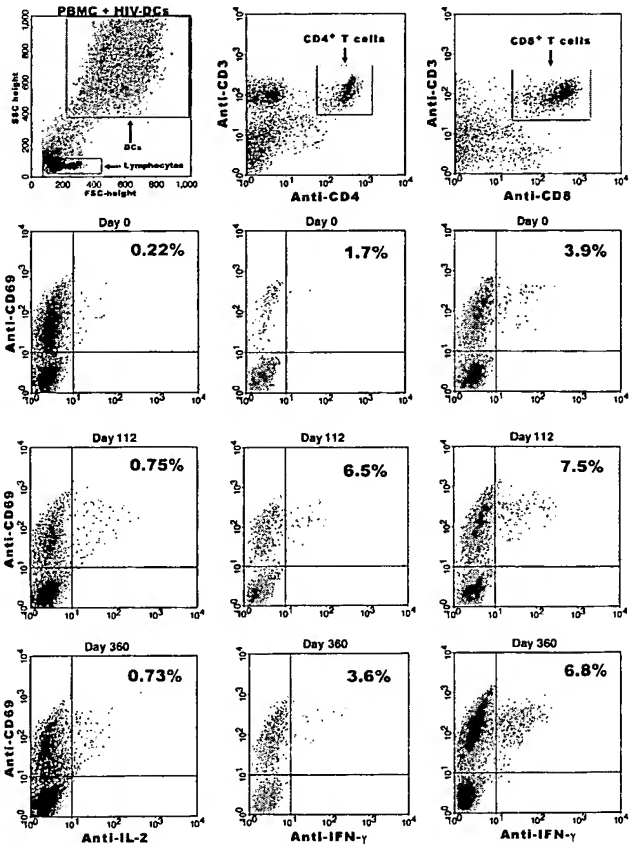

Figure 2 Intracellular cytokine detection of T cells following stimulation with AT-2-inactivated HIV-1-pulsed DC (patient 13). The data represent the percentage of total T cells secreting IL-2 or IFN-γ. For the bottom three rows of graphs, the left column and middle columns represent data for CD4$^+$ T cells secreting IL-2 or IFN-γ, respectively, and the right column represents data for CD8$^+$ T cells secreting IFN-γ. The monoclonal antibody isotype controls for IL-2 or IFNγ were less than 0.02% and 0.05% respectively. The background levels of IL-2-expressing CD4$^+$ T cells, IFN-γ-expressing CD4$^+$ T cells and IFN-γ-expressing CD8$^+$ T cells in the presence of nonpulsed DCs alone were less than 0.01%, 0.01% and 0.03% respectively.

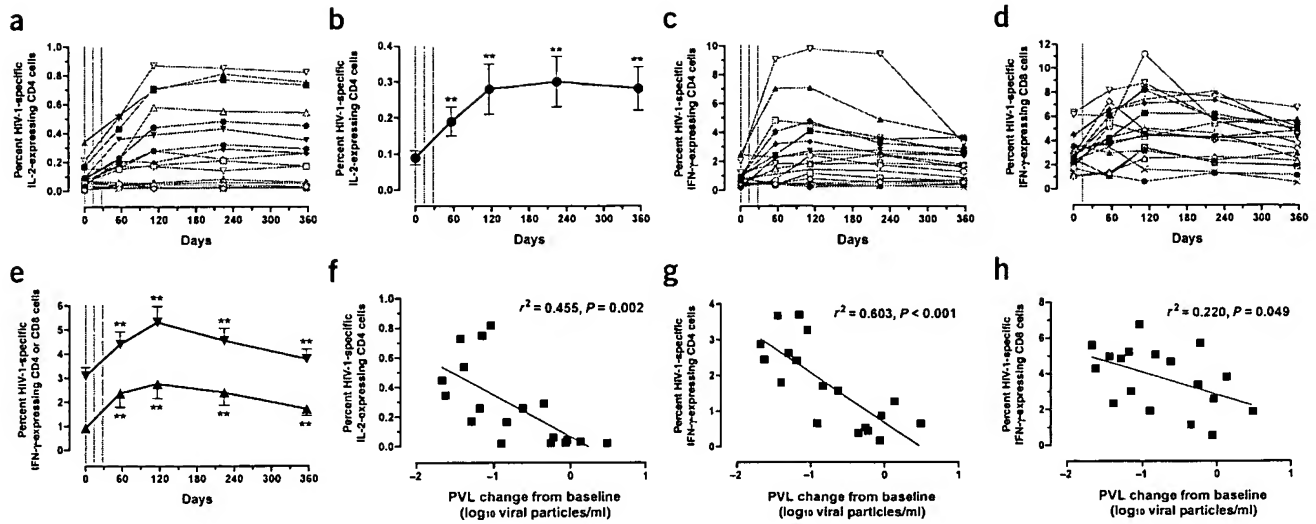

Figure 3 HIV-1-specfic T-cell immunity in the 18 immunized patients. (a,b) Individual or mean (± s.e.m) (solid circles) HIV-1-specific IL-2-expressing CD4+ T cells at baseline and after immunization. (c) Individual HIV-1-specific IFN-γ-expressing CD4+ T cells. (d) Individual HIV-1-specific IFN-γ-expressing CD8+ T cells. (e) Mean (± s.e.m) HIV-1-specific IFN-γ-expressing CD4+ (upward-facing triangles) or CD8+ (downward-facing triangles) T cells. (f,g) Correlation between 1-year changes of PVL from baseline and HIV-1-specific IL-2-expressing or IFN-γ-expressing CD4+ T cells by 1 year. (h) Correlation between HIV-1-specific IFN-γ-expressing CD8+ T cells by 1 year and 1-year changes of PVL from baseline. $^{**}P < 0.01$.

cacy of such a therapeutic vaccine will not be definitively proven until a randomized trial with an appropriate control arm has been performed.

The observation that, at 1 year, the percentage of HIV-1-gag-specific CD8+ T cells expressing perforin was positively correlated with the 1-year PVL decline (Fig. 5f) underscores the major role of perforin-expressing effectors in controlling HIV-1 replication *in vivo*. This is in keeping with the observation that perforin-expressing HIV-1-specific CD8+ T cells were associated with the partial control of viral replication in some acutely infected patients with structured antiviral treatment interruption as well as in untreated long-term nonprogressors[19]. These findings suggest that intracellular perforin staining, coupled with existing assays for antigen-specific effectors (such as HLA-restricted tetramer binding technique or ICC assay), could be the most sensitive surrogate markers to monitor functional HIV-1-specific CTL activity *in vivo*. In addition, the significant correlation between viral suppression and durable increase in frequencies of HIV-1-specific CD4+ T_H1 cells and CD8+ effectors observed in immunized patients (Figs. 3 and 5) favors the notion that a strong virus-specific CD4+ T_H1-cell response is required to enable virus-specific CD8+ effectors to contain HIV-1 replication *in vivo*. This is in agreement with the findings from several studies showing a correlation between high levels of virus-specific CD4+ T cells and the (spontaneous) control of viral replication in the chronic phase of HIV-1 infection[4–6,20] as well as during the course of human hepatitis virus B or C infection[21–25]. In this regard, virus-specific CD4+ T_H1 cells may provide help to promote virus-specific CD8+ T-cell differentiation (such as perforin expression) through a direct mechanism by secreting T_H1 cytokines *in situ* and/or through an indirect mechanism by triggering CD40-mediated DC activation which in turn favors CD8+ T-cell differentiation[26]. Vaccines boosting virus-specific CD4+ T cells could thus be promising strategies for treating people with progressive HIV-1 infection and other chronic viral diseases.

Given that stronger 1-year PVL decreases following the DC vaccination were associated with higher baseline CD4 cell counts (Supplementary Fig. 2 online) or HIV-1-specific IL-2-expressing CD4+ T cells (Supplementary Fig. 2 online), which decline progressively along the course of the infection, it is conceivable that an early therapeutic vaccine intervention could increase the probability of achieving sustained viral suppression. This notion is also supported by the dramatic viral suppression that we observed in immunized Chinese macaques with early chronic simian immunodeficiency virus (SIV) infection[18].

Considering the humoral arm of the immune response after vaccination, it is interesting to observe that our inactivated whole HIV-1-loaded DC vaccine did not induce any neutralizing antibodies. This is in keeping with the consensus that there is poor neutralizing antibody response

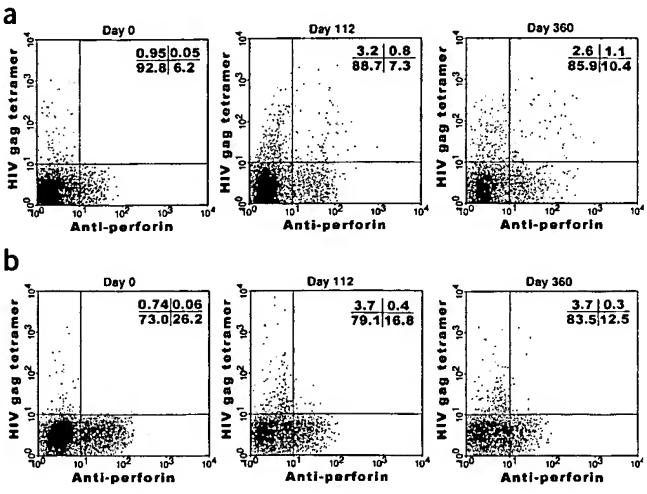

Figure 4 HIV-1-gag tetramer staining and intracellular perforin detection in gated CD3+CD8+ cells. (a) Patient 13. (b) Patient 19. The data represent the percentage of gag-tetramer-specific and/or perforin-specific staining cells. The nonspecific tetramer staining of normal donor cells was less than 0.02% and the isotype controls for anti-perforin monoclonal antibodies were less than 0.03%.

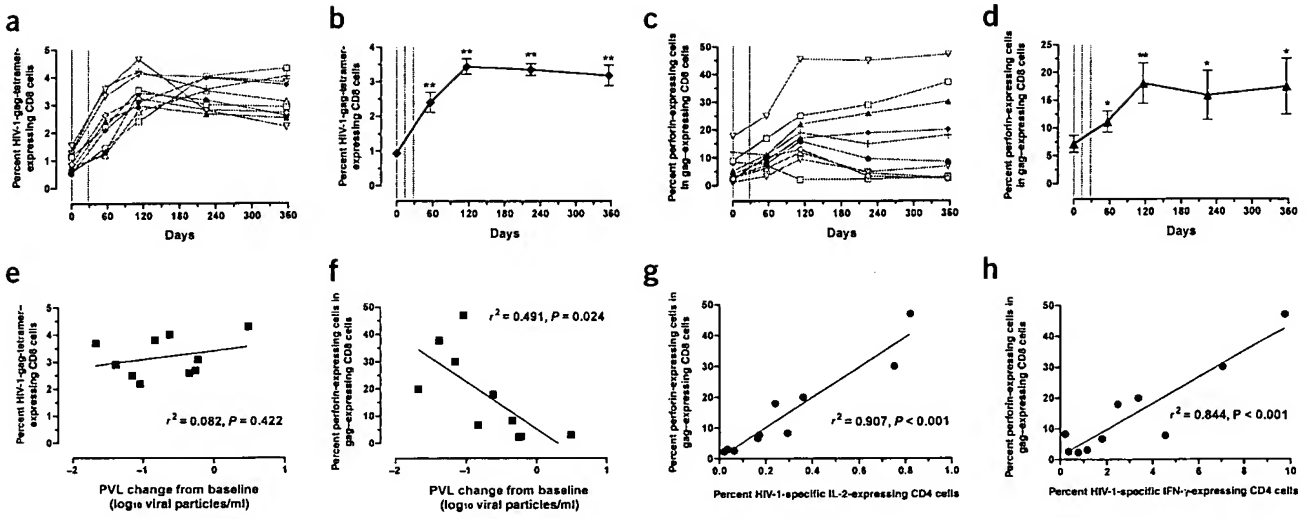

Figure 5 HIV-1 gag–specific perforin-expressing CD8+ T cells (effectors) in 10 immunized HLA A*0201-positive individuals. (a,b) Individual or mean (± s.e.m.) (solid diamonds) HIV-1-gag-tetramer-positive CD8 cells at baseline and after immunization. (c,d) Individual or mean (± s.e.m) (solid triangles) HIV-1 gag tetramer–expressing CD8 cells expressing perforin. (e,f) Correlation between 1-year changes of PVL from baseline and HIV-1 gag tetramer–expressing CD8 cells or HIV-1 gag tetramer–expressing CD8 cells expressing perforin by 1 year. (g,h) Correlation between HIV-1 gag tetramer–expressing CD8 cells expressing perforin by 1 year and HIV-1-specific IL-2-expressing or IFN-γ-expressing CD4 cells by 1 year. $*P < 0.05$; $**P < 0.01$.

during the course of HIV infection[20]. We had however observed an increase in the neutralizing antibody response following AT-2-SIV-loaded DC immunization in SIV-infected macaques[18], This discrepancy might be explained by the fact that in the macaque study the use of *in vitro* transformed CEMx174 cell lines and cell line–adapted SIVmac251 for detecting neutralizing antibodies could have artificially resulted in serum neutralization titers much higher than those obtained in humans using an assay system involving primary peripheral blood mononuclear cells (PBMC) and primary viral isolates[27–29]. The stable serum levels of total antibodies specific for HIV-1 in immunized individuals (Supplementary Table 5 online) might represent either saturation of anti-HIV-1 antibody-producing capacities or an unresponsiveness resulting from chronic B-cell hyperactivity with impaired antigen-specific humoral immunity as observed in HIV-infected patients[30–32].

An important question in vaccinology concerns the best antigen source (inactivated whole virus or recombinant proteins or peptides) for generating a protective anti-HIV-1 cellular immunity. Our results suggest that the adoptive transfer of inactivated whole virus–pulsed DCs is an excellent antigenic preparation to expand and activate *in vivo* virus-specific CTLs with a wide range of potential HLA-restricted epitopes. Up to 5.3% (mean) of HIV-1-specific effector memory (IFN-γ-expressing) CD8+ T cells were observed 112 d after the immunization (Fig. 3e) whereas only 3.5% (mean) of total CD8+ T cells were stained by gag tetramer at the same time point (Fig. 5b). Given that only 10% of gag tetramer–staining CD8+ T cells have been reported to express IFN-γ following gag-specific stimulation[33,34], it seems that more than 90% ([5.3% – 0.35%] ÷ 5.3% = 93.4%) of HIV-1-specific memory CD8+ T cells expanded by the inactivated whole virus–pulsed DCs could probably be attributed to HIV-1 antigenic determinants other than gag. In this setting, it has been recognized that AT-2-inactivated SIV/HIV enters the DC through a receptor-mediated mechanism[35,36] eliciting a potent HLA-I-restricted CTL response[37,38], whereas recombinant viral proteins enter DCs through nonspecific endocytosis inducing preferentially humoral (antibody) response. The processing of AT-2-inactivated SIV/HIV virion antigens by DCs thus probably differs from that of recombinant proteins in terms of antigenic epitope presentation. Taken together, these differences might explain why an earlier pilot trial with DCs pulsed with a limited number of recombinant HIV-1 antigens or peptides did not have any effect on viral load or CD4 cell count[39].

Because our vaccine was made of inactivated whole virus-loaded DCs that were matured with cytokines *in vitro*, a pertinent question was whether the viroimmunologic response following the DC vaccination depends on the levels of DC maturation in the final vaccine preparation. In the present study, the majority of participants' monocyte-derived DCs (51–81%) underwent maturation (CD83 expression) following *in vitro* cytokine-induced stimulation (Supplementary Table 1 online). This is in agreement with earlier reports showing that monocyte-derived DCs from HIV-1-infected individuals remained functionally intact and capable of priming naive T cells or stimulating memory T cells *in vitro*[40–42]. The lack of correlation between the number of mature DCs and viral-load responses (Supplementary Fig. 2 and Supplementary Table 1 online) allows us to reject the hypothesis that a difference in DC maturation might account for different viral-load responses in immunized individuals. Our final DC preparations contained 2–28% small cells that were mostly T and B cells. Although the potential presentation of the inactivated whole HIV-1 by B cells cannot be ruled out, involvement of B cells is unlikely because currently there is no evidence that HIV can enter human B cells. Moreover, a recent study in the macaque model has shown little involvement of B cells in the presentation of AT-2-inactivated SIV[43].

Vaccines for treating chronic HIV-1 infection are distinct from prophylactic vaccines against HIV-1. The goal of therapeutic vaccination is the induction of strong and durable cellular responses that can control viral replication established for years in lymphoid tissues. The ultimate goal is to sustainably reduce the viral load of HIV-1-infected individuals to as low a level as possible. This reduction would protect these individuals from disease progression, which would allow them to live without harmful and expensive daily antiretroviral drugs. At the same time, this would minimize their risk of sexually transmitting the virus to healthy people[44].

METHODS

Participants. The National Ethical Committee of the Brazilian Ministry of Health approved this phase 1-2 trial. HIV-1-positive volunteers gave written informed consent before being enrolled in this study. The following criteria had to be met 3 months before the first vaccination: age of ≥18 years, absence of pregnancy, HIV-1 seropositivity for ≥1 year, no clinical AIDS, absence of antiretroviral therapy for ≥6 months, hemoglobin ≥10 g/dl, platelets ≥100,000 cells/μl, blood $CD4^+$ cells ≥300 cells/μl, plasma viral RNA ≥10,000 copies/ml and absence of other chronic diseases. Initially, 20 chronically HIV-1-infected participants were enrolled in this study. Two participants were excluded before being vaccinated: one because his baseline plasma viral load was below 5,000 copies/ml, as determined by repeated viral-load measurements; the other because she had a platelet count below 100,000 cells/μl at day 0. A total of 18 participants were thus included in the present study.

Vaccine. Autologous HIV-1 isolates, which were obtained from CD8-depleted PBMCs by viral culture, were inactivated by AT-2 (Sigma) as described[37] under GMP (good manufacturing practices) conditions. The complete inactivation of each of the viral isolates was confirmed by the negative results of a 1-month culture in HIV-negative donor PBMCs. One week before immunization, $1–3 \times 10^{10}$ PBMCs were collected by a 3-h leukapheresis from each patient and then transferred to a biosafety (level P3) clean room for a standardized 7-d culture[18]. Briefly, we subjected freshly collected PBMCs to plastic adherence at a density of 1×10^6 cells/cm^2 in the presence of 0.5% of clinical-use human serum albumin (Laboratoire Français du Fractionnement et des Biotechnologies). After a 2-h incubation at 37 °C in 5% CO_2, we removed nonadherent cells by rinsing with sterile PBS buffer. Adherent cells were then cultured for 5 d in clinical grade CellGro DC medium (CellGenix) containing 2000 U/ml GM-CSF (Schering-Plough) and 50 ng/ml IL-4 (CellGenix). At day 5, we exposed 6×10^7 DCs to AT-2-inactivated autologous virus (1×10^9 viral particles/ml) at 37 °C for 2 h and froze the remaining nonpulsed DCs in liquid nitrogen. After two washes to remove free virus, cells were cultured for an additional 2 d in the DC medium supplemented with clinical-grade cytokines IL-1β (10 ng/ml, CellGenix), IL-6 (100 ng/ml, CellGenix), and TNF-α (50 ng/ml, CellGenix). At day 7, we performed quality control with flow cytometry; 3×10^7 quality control–approved viable DCs were resuspended in 1 ml of sterile 0.9% chloride sodium solution and were ready for injection. Each patient received four subcutaneous injections of 0.25 ml in close proximity to left and right axillary and inguinal areas. At weeks 2 and 4, we administered two booster injections with the same number of viable virus-loaded autologous DCs. These DCs were processed from the remaining frozen nonpulsed DCs, which were pulsed with AT-2-inactivated HIV-1 upon thawing just prior to the last 2-d culture.

Viral load assays. We measured plasma HIV-1 RNA using the Monitor kit (Roche Diagnostics). Cell-associated HIV-1 DNA or supernatant HIV-1 RNA was quantified as previously described[37,45].

Anti-HIV-1 and neutralizing antibody assays. We determined serum titers of antibody specific for HIV-1 using a limiting dilution assay with a commercial ELISA kit (GENSCREEN HIV1/2 version 2, Bio-Rad). The titers were determined as reciprocals of the last serum dilutions showing a ≥50% positivity by ELISA. We performed neutralization against infection of healthy donor PBMCs by autologous virus in participants' sera as described[18]. Control cells were exposed to the same infectious dose of autologous virus or to the same virus dose pretreated with the sera taken from five healthy donors.

Flow cytometry assays. Intracellular IL-2- and IFN-γ-expressing $CD4^+$ T cells or IFN-γ-expressing $CD8^+$ T cells following *ex vivo* stimulation with autologous DCs pulsed with AT-2-inactivated autologous virus were detected by previously described ICC assay[2,4]. The ICC kits and all monoclonal antibodies (including corresponding isotype controls) for the quality control of DCs were purchased from BD Bioscience Europe. We determined cell viability by intracellular DNA staining with propidium iodide (20 μg/ml, Sigma). AT-2-inactivated HIV-1-loaded autologous DCs used as stimulating cells for ICC assay were the same as those used for the booster immunizations. We measured HIV-1-specific $CD8^+$ effector T cells using a combination of HLA-A*0201 HIV gag (amino-acid sequence: SLYNTVATL) tetramer-phycoerythrin (Beckman Coulter) and monoclonal antibodies specific for perforin-FITC, CD8-PerCP-Cy5.5 and CD3-APC (BD Biosciences Europe). The four-color flow cytometry studies were performed on FACSCalibur (BD Biosciences). We determined the specific staining by subtracting the nonspecific staining of isotype controls and determined the gag-tetramer-specific staining by subtracting the background tetramer binding in normal HLA A*0201 donor T cells.

Statistical analysis. Paired data before and at different time points after immunization were compared by the Wilcoxon test.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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